

THE aetiology of Alzheimer's disease (AD), which affects a large proportion of the aged population is unknown and the treatment unresolved. The role of beta amyloid protein (β A4), derived from a larger amyloid precursor protein (APP) in AD is the subject of intense research. Here I report observations that in 14 autopsy cases with histopathologically confirmed AD, spirochetes were found in blood and cerebrospinal fluid and, moreover, could be isolated from brain tissue. Thirteen age-matched control cases were without spirochetes. Reference strains of spirochetes and those isolated from brains of AD patients, showed positive immunoreaction with monoclonal antibody against the β amyloid precursor protein. These observations suggest that spirochetes may be one of the causes of AD and that they may be the source of the β amyloid deposited in the AD brain.

Key words: Alzheimer's disease; Amyloid; Amyloid precursor protein; β amyloid protein; Spirochetal disease

Alzheimer's disease— a spirochetosis?

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Introduction

Alzheimer's disease (AD)¹ is characterized by a slow, progressive decline of cortical functions, particularly cognition and memory. The most consistent histological changes are localized in the cerebral cortex: atrophy due to neuronal loss, senile plaques (named also argyrophylic or neuritic plaques), neurofibrillary tangles and astrocytic and microglial proliferation. Fibrillar amyloid proteins are deposited in the leptomeningeal and cortical vessel walls and in senile plaques.^{2,3}

The major protein subunit of the amyloid fibril is the beta amyloid protein (β A4), a small, self-aggregating polypeptide of about 4 kDa² which is derived from a larger, transmembrane amyloid precursor protein (APP).⁴ Molecular and biochemical studies have shown that an excess of β A4 is the primary event in AD, but its source until now has not been established (for a review see reference 5).

Dementia associated with cortical atrophy and microgliosis has also been observed in the late stages of two spirochetal diseases: Lyme disease—a late stage of neuroborreliosis—caused by *Borrelia burgdorferi*^{6,7} and general paresis—tertiary stage of neurosyphilis—caused by *Treponema pallidum*. Two cases of concurrent neocortical borreliosis and AD have been reported by MacDonald and Miranda.^{8,9} The authors suggest a possible association between *Borrelia burgdorferi* and AD. A careful study, using several methodological approaches, of 18 AD cases failed to support an association between *Borrelia burgdorferi* and AD, but the authors did not rule out the possibility that another spirochete, not detectable by their methods, may be responsible for AD.¹⁰

Before the era of serological tests the examination of infected material by dark field microscopy was the

laboratory procedure of choice for the diagnosis of spirochetal infection. Therefore an attempt to demonstrate spirochetes in AD using this method was the aim of this study. But if, indeed, spirochetes play a role in the pathogenesis of AD, how may this be brought in harmony with the excess of β A4 in the brain reported to cause the manifestations of AD? If spirochetes are found to be immunoreactive with antibodies against APP, these microorganisms may be the source of the β A4.

Materials and Methods

Twenty-seven randomly chosen autopsy cases with various clinical diagnoses were investigated. Autopsy cases with the clinical diagnosis of Alzheimer's dementia, were always taken in the series. The only criterion in selecting cases without Alzheimer's dementia, was that of the age of the patients in the aim to have age-matched control cases for the AD patients (see Table 1). Blood was taken by arterial or intracardiac puncture, and CSF by cisternal puncture. Both procedures were performed under sterile conditions. Blocks (ca 3 × 3 × 1 cm) from frontal, temporal and inferior parietal cortical regions were removed and immediately frozen at -80°C . The timespan between death and autopsy varied from 6 to 16 h. After removing these samples, the brains were fixed in 10% formalin for about one month and processed for routine neuropathological examination and for the systematic investigation of histological signs of AD. The neuropathological diagnosis of AD was based not only on observations made in sections that were silver-stained for AD,¹¹ but also on the immunohistochemical detection of β A4 using specific antibody (DAKO,

Table 1. Demonstration of spirochetes by dark field microscopy in the blood, CSF and those isolated from the cortex and cultured from the blood in 27 autopsy cases, including 14 cases with Alzheimer's disease (AD)

Neuropathological diagnosis	Age	Blood	CSF	Spirochetes isolated from the cortex	Spirochetes cultured from blood
1. AD	74	+	+	+	+
2. AD	79	+	+	+	+
3. AD	86	+	+	+	+
4. AD	78	+	+	+	+
5. AD	76	+	+	+	-
6. AD	78	+	+	+	0
7. AD	89	+	+	+	0
8. AD	73	+	+	+	0
9. AD	64	+	+	+	0
10. AD	72	+	+	+	0
11. AD	97	+	+	+	0
12. AD	88	+	+	+	0
13. AD	81	+	+	+	0
14. AD	74	+	+	+	0
15. Glioblastoma	64	-	-	-	-
16. Subependymoma	78	-	-	-	-
17. Hypertensive encephalopathy	86	-	-	-	-
18. Hypertensive encephalopathy	83	-	-	-	-
19. Hypertensive encephalopathy	84	-	-	-	-
20. Wernicke's encephalopathy	72	-	-	-	0
21. Cerebral hypoxia	91	-	-	-	0
22. Cerebral contusion	72	-	-	-	0
23. Cerebral infarct	82	-	-	-	0
24. Cerebral infarcts	79	-	-	-	0
25. Atheromatosis	64	-	-	-	0
26. Atheromatosis	82	-	-	-	0
27. No cerebral lesion	62	-	-	-	0

+: presence of spirochetes; -: no spirochetes; 0: not investigated.

M872). The immunohistochemical biotin-avidin peroxidase complex technique was used. A silver method designed for the detection of spirochetes¹² was applied to paraffin or to frozen sections.

Examination of the blood and CSF was carried out in all cases using dark field microscopy. 10 µl of blood diluted with 20 µl of sterile distilled water was put on a slide, coverslipped and examined by dark-field microscopy using a ×100 immersion objective. The same procedure was carried out with 30 µl (undiluted) CSF. In each case, several samples were carefully and repeatedly examined. For the isolation of the spirochetes¹³ small fragments of the cerebral cortex, with a volume of about 1 cm³, were taken from sterile *post mortem* brain biopsy material. They were finely sliced with a sterile surgical blade and put into a 10 ml sterile tube to which 3 ml of sterile physiological NaCl or PBS was added. After continuous shaking for about 30 min 30 µl of the supernatant was put on a slide, coverslipped and examined in dark field illumination. The modified Noguchi medium¹⁴ used for the cultivation of spirochetes consisted of a mixture of 6 ml of sterile PBS, 2 ml of sterile distilled water and 1 ml foetal calf serum. After inoculation of 1 ml blood taken at autopsy, it was covered with sterile paraffin oil and incubated at room temperature.

For ultrastructural analysis small fragments of corti-

cal sections that were silver-stained for AD¹¹ as well as fragments of immunostained unfixed frozen sections using a monoclonal antibody against APP (Boehringer, 1285262, dil. 1:5) were embedded in epon. The ultrathin sections from the silver-stained sections which were not contrasted, and the immunostained sections contrasted with uranyl nitrate and lead citrate, were examined in a Philips CM-10 electron microscope.

To study whether spirochetes may be the source of the excess of βA4 deposited in the brain, smears of the Nichols strain of *Treponema pallidum*, the B31 strain of *Borrelia burgdorferi* and those isolated from the cortex of the AD cases were immunostained with an APP monoclonal antibody (Boehringer, 1285262) specific to the N-terminal region of all APP isoforms.¹⁵ One may argue that the silver impregnation technique designed for the demonstration of spirochetes is not entirely specific. Therefore an additional case of concurrent AD and serologically confirmed Lyme disease was used to investigate immunohistochemically whether *Borrelia burgdorferi* was present in brain tissue. Here it was assumed that *Borrelia burgdorferi* may also cause AD. If true, then a specific antibody against *Borrelia burgdorferi* would stain the accumulation of spirochetes in the cortex. The presence of spirochetes would allow the comparison in the same brain of the localizations of spirochetes, of APP and of βA4. For the demonstration of the spirochetes the immunoreactions were carried out on acetone-fixed frozen sections using a monoclonal antibody against *Borrelia burgdorferi* (Biosdesign, C63780M). The immunohistochemical reaction for APP (Boehringer, 1285262) was also carried out on acetone-fixed frozen sections. For the demonstration of β amyloid (DAKO, M872) formalin-fixed frozen or paraffin sections were also used. As a negative control all immunostaining procedures were carried out with the omission of the primary antibody. As a positive control for the immunohistochemical demonstration of spirochetes, smears of the reference strain of *Borrelia burgdorferi* were used.

Results

In the blood and the CSF from 14 out of the 27 cases, motile, coiled spirochetes were observed (Table 1; Fig. 1A, B). Their diameter was approximately 0.2 to 0.3 µm and their length varied between 8 and 30 µm. A rough estimate of the number of spirochetes per cc of blood was 100 to 400; and per cc of CSF, 50 to 200. In the remaining 13 cases spirochetes were observed neither in the blood nor in the CSF (Table 1). Severe histological and immunohistochemical changes typical of AD were found in all 14 cases whose blood and CSF contained spirochetes (Fig. 3). An astrocytic and a microglial proliferation particularly in association with senile plaques were also observed (Fig. 3E, F).

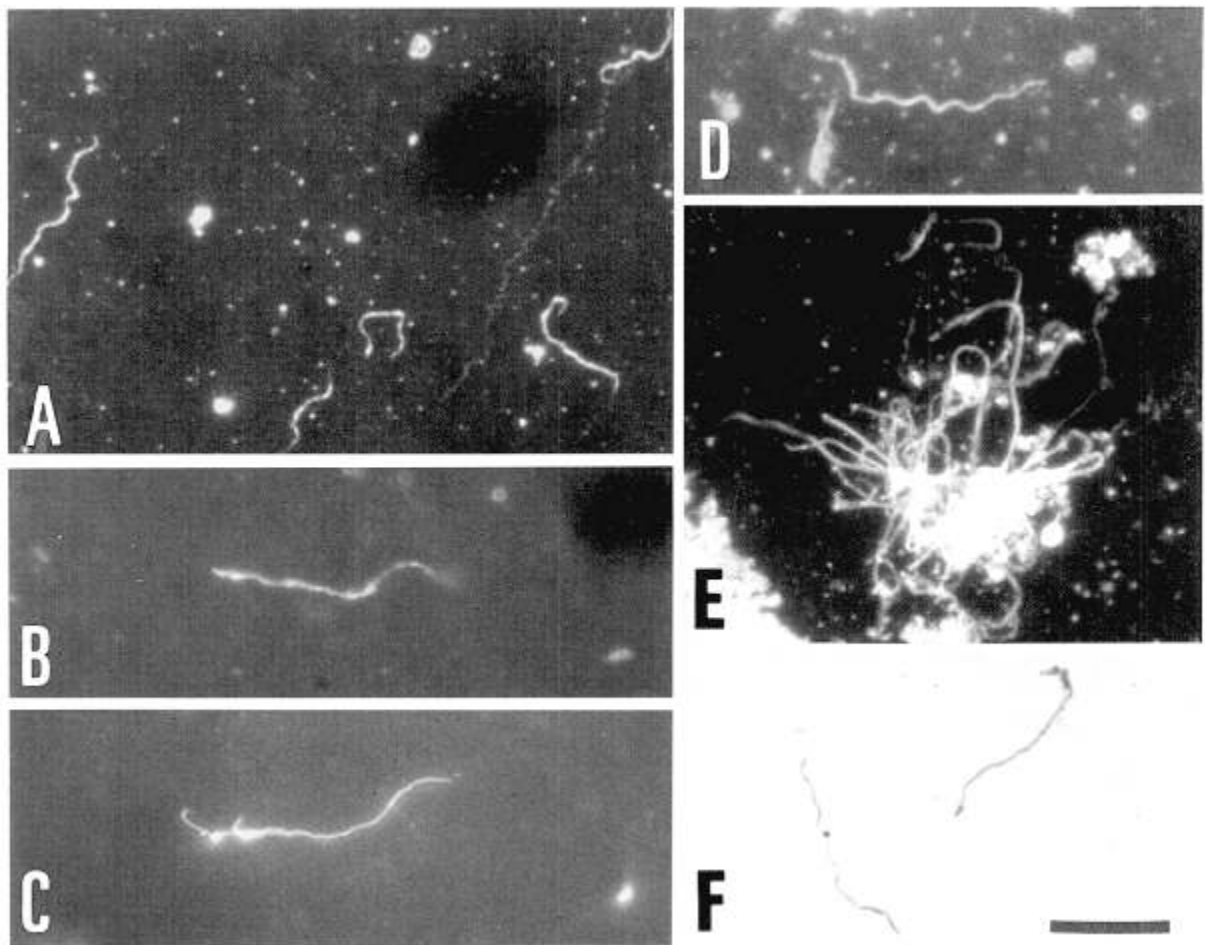


FIG. 1. **A** and **B**: Dark field photomicrographs showing spirochetes in the CSF in two autopsy cases of Alzheimer's disease (AD). **C**: Smear of *Borrelia burgdorferi* (B31 strain) immunostained with a fluorescein conjugated rabbit polyclonal antibody against this spirochete (Biodesign, B65303F, dil.1:50). **D** and **E**: Dark field photomicrographs showing the morphology of spirochetes isolated from the cortex of two AD cases. **F**: Spirochetes from the same strain as shown in **C** which were immunoreactive with an antibody against APP (Boehringer, 1285262 dil.1:5), biotin-avidin peroxidase complex technique. As a negative control the reaction was carried out with omission of the primary antibody (not shown). Bar for A-F is shown in F and corresponds to 10 μ m.

When using a silver method for the demonstration of spirochetes,¹² the accumulations of the silver-stained spirochetes are, both in architecture and in pattern of distribution, the perfect resemblance of senile plaques (Fig. 3B). There were no perivascular lymphocytic cuffs, microglial nodules, or signs of meningitis in these 14 AD cases. Leptomeningeal fibrosis and granular endependymitis were often observed.

In the remaining 13 cases, those without spirochetes in the blood and CSF, the neuropathological examination revealed no signs of AD, but several other pathologies, including hypertensive, hypoxic or traumatic brain damage and Wernicke's encephalopathy due to chronic alcoholism. In two cases a brain tumour (subependymoma and glioblastoma) was found (see Table 1). In the silver- and immunostained sections for AD there were no senile plaques or neurofibrillary tangles in the cortex of these 13 control cases.

Spirochetes were isolated from the unfixed cortical tissue from all 14 AD cases (Table 1; Fig. 1D, E). The rough estimate of the number of spirochetes per cc of

cortex was 1000 to 2000. The number of microorganisms in the cortex is probably underestimated because not all spirochetes were detached from the cortical fragments and therefore were not clearly visible in the isolation fluid. From the cortex of three out of four AD cases tested, spirochetes were cultured with success in a synthetic (BSK) medium. No spirochetes were found in the cortex from the 13 cases without AD (Table 1). In addition, spirochetes were cultured from the blood, in four out of five arbitrarily chosen AD cases, in a modified Noguchi medium (Table 1). The spirochetes not only survived but also proliferated markedly after 2 weeks of incubation. The rough estimate of the number of spirochetes per 1 cc of culture medium was between 2000 and 8000, i.e. an increase of about $\times 20$ with respect to the initial samples taken from the blood. No spirochetes could be cultured from the blood in five cases tested without AD (Table 1).

The ultrastructural study of silver-stained and anti-APP-immunostained sections of the cerebral cortex in three AD cases tested also revealed coiled elements

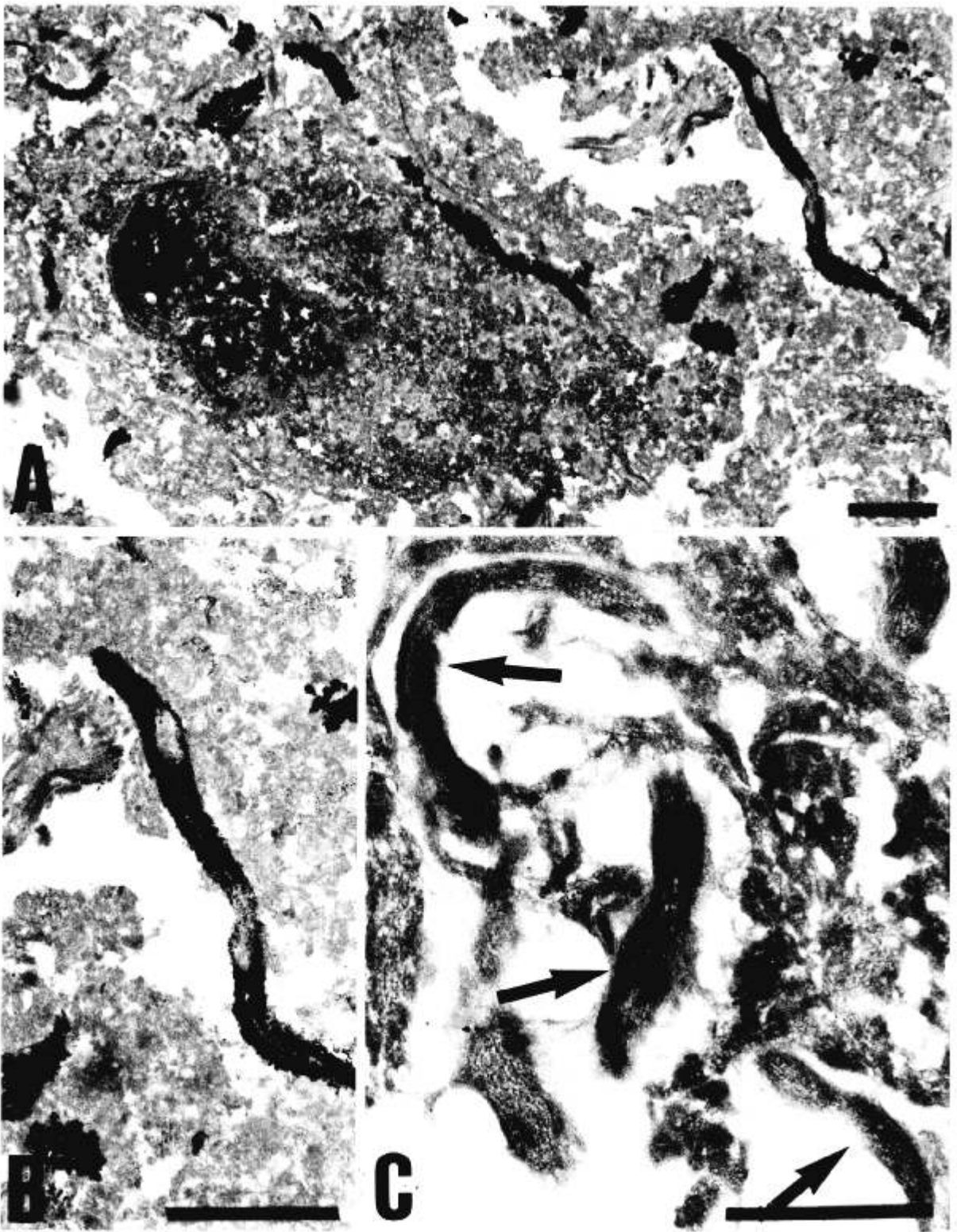


FIG. 2. **A:** Electron micrograph from an ultrathin section prepared from a frozen section, which had been silver-stained for senile plaques¹¹ in an AD case. Illustration shows silver deposits within elongated elements morphologically compatible with spirochetes. **B:** The silver-impregnated element lying at the right hand side of **A** shown at higher magnification. **C:** Electron micrograph from an immunostained frozen section in an AD case. Immunostaining was carried out using a monoclonal antibody against APP. Arrows point to elongated elements compatible with fragments of spirochetes localized in a senile plaque. The material shown in **A–C** derives from parietal cortex. Bars in **A–C** represent 1 μm .

whose morphology was compatible with that of spirochetes (Fig. 2A–C). Smears of reference strains of *Treponema pallidum* (Nichols strain), of *Borrelia burgdorferi* (B 31 strain) and smears of spirochetes isolated

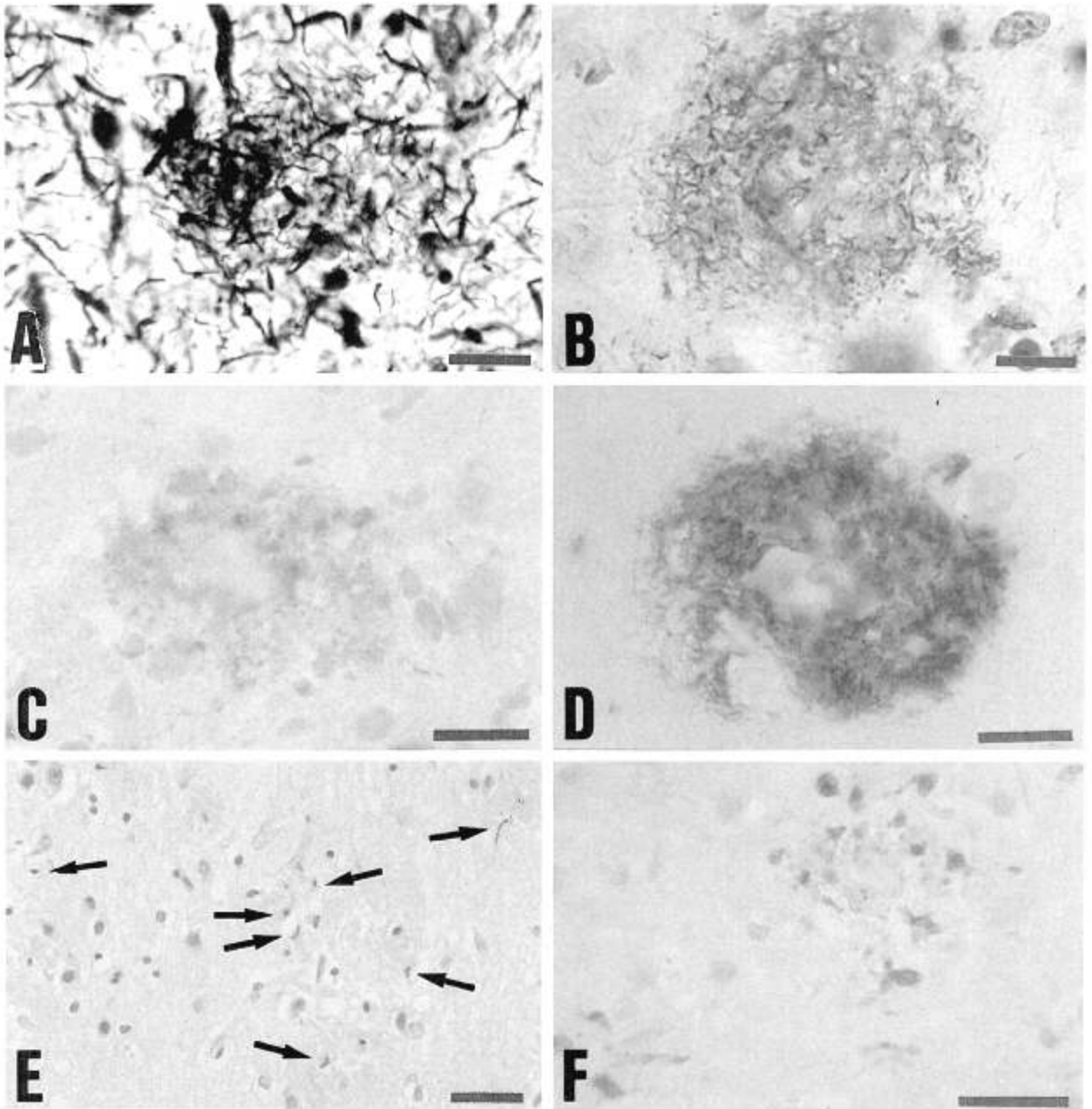


FIG. 3. Photomicrographs taken from the inferior parietal cortex of the brain from the 14 AD cases investigated in this study. **A:** Frozen section stained with a silver-technique for AD¹¹ showing senile plaque. For **B** a technique designed for the visualization of spirochetes¹² was used. The accumulation of silver-stained spirochetes follows the pattern of the senile plaque. **C** and **D:** Cryostat sections fixed in acetone and immunostained with a monoclonal antibody against APP (Boehringer, 1285262, dil.1:5) and β amyloid (DAKO, M872), respectively, showing senile plaques. **E:** Paraffin section stained with haematoxylin and eosin. Arrows point to putative reactive microglia. **F:** Cryostat section stained with a monoclonal antibody against human monocytes and macrophages (DAKO, CD68 PGM1: M718) showing microgliosis, particularly in association with senile plaques. Bars in A–D correspond to 20 μ m, and in E and F to 50 μ m.

from the AD cases, showed a positive immunoreaction, using a monoclonal antibody against APP (Fig. 1F). The negative controls which were carried out with the omission of the primary antibody were always negative.

In an additional case with concurrent AD and Lyme

disease, using a specific antibody against *Borrelia burgdorferi*, spirochetes were found in senile plaques (Fig. 4B, D), in the leptomenigeal and cortical vessel walls, in neurones (Fig. 4E, F), and in microglial cells. They were also found as solitary elements in the neuropil (Fig. 4G). The location of spirochetes was similar to

that of the immunostained APP and β A4 accumulations (Fig. 4A–D), and follows the pattern of the senile plaques. The neurofibrillary tangles were also immunoreactive with the antiserum against *Borrelia burgdorferi*. Sections in which the immunoreaction was carried out with the omission of the primary antibody did not show spirochetes.

Discussion

There is evidence suggesting an infectious aetiology for AD. Cases of familial AD can be transmitted to the chimpanzee in the form of spongiform encephalopathy, resembling Creutzfeldt-Jakob disease (CJ).¹⁶ In addition, the inoculation of experimental animals by

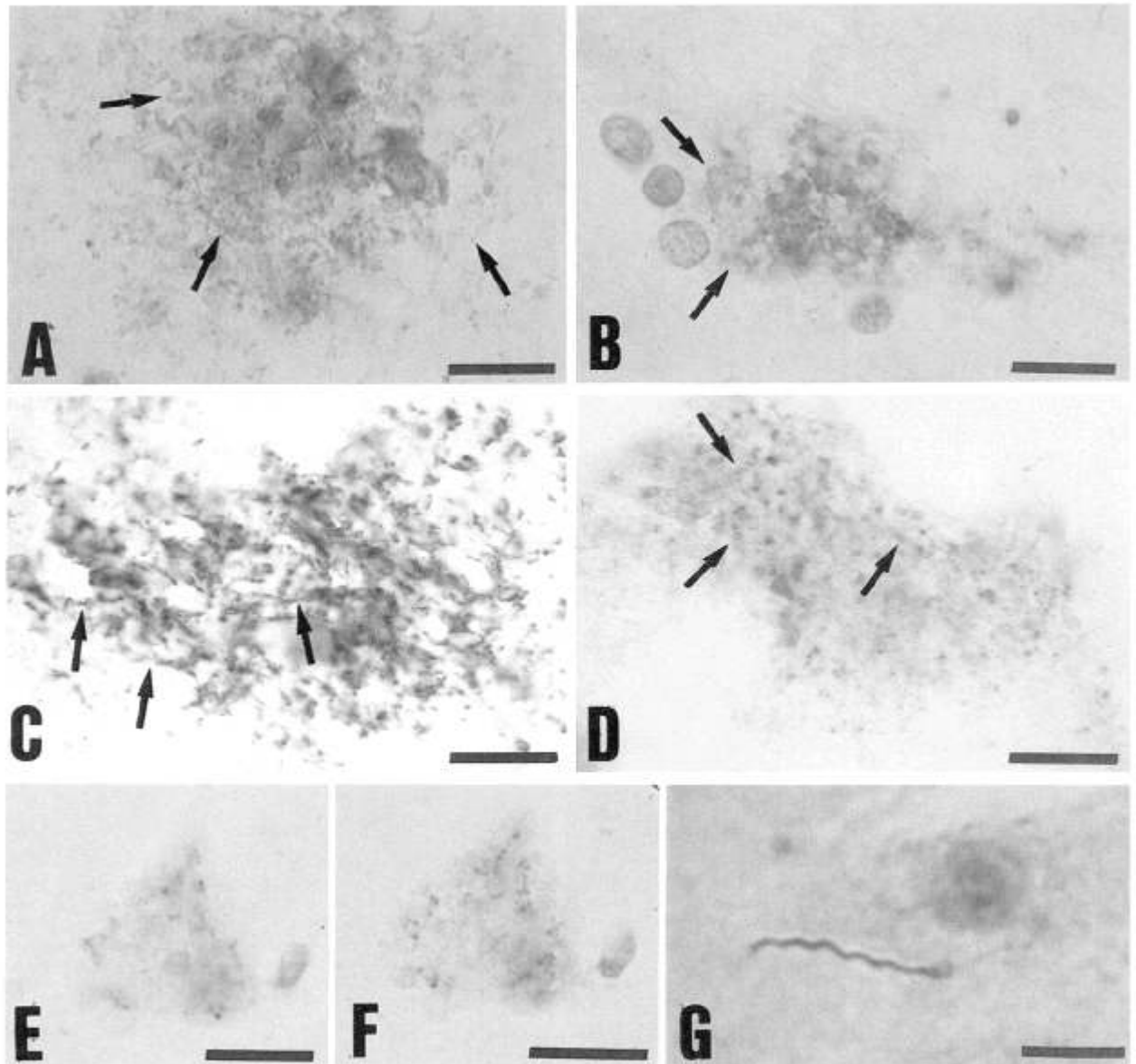


FIG. 4. Photomicrographs documenting the immunohistochemical findings of a case with concurrent AD and Lyme borreliosis, showing similar accumulation of APP, β A4 and spirochetes in the cerebral cortex. The biotin-avidin peroxidase complex technique was used, preparations were counterstained with haematoxylin. **A:** Cryostat section fixed in acetone and immunostained with anti-APP monoclonal antibody (Boehringer, 1285262, dil.1:5) showing fine, coiled elements (arrows) within a senile plaque. **B:** Cryostat section fixed in acetone and immunostained using monoclonal antibody against *Borrelia burgdorferi* (Biodesign, B65307B, dil.1:50). **C and D** show immunostained filaments (arrows) in subpial plaques when using monoclonal antibodies against β A4 (DAKO, M872) in a formalin-fixed paraffin section (C) and against *Borrelia burgdorferi* (D). **E and F** show the same neurone photographed at different levels. The nucleolus in E helps to identify the cell in question as a neurone, while in F filamentous elements immunoreactive with a monoclonal antibody against *Borrelia burgdorferi* (Biodesign, B65307B, dil.1:50) are seen. **G:** shows a solitary coiled immunostained spirochete in the neuropil. Bars in A–F correspond to 20 μ m, and in G to 10 μ m.

the scrapie agent may induce the formation of senile plaques, morphologically similar to, but biochemically distinct from, AD plaques.¹⁷ Masters *et al*¹⁸ suggested that there are sufficient similarities between the amyloid filaments and proteins in AD and in scrapie to suspect that they are derived by mechanisms which have in common the generation of self aggregating polypeptides of low molecular weight. The authors' comment that if the scrapie filaments and proteins in CJ are an integral part or a direct effect of the infectious agent, it follows that AD is also an infectious process similar to scrapie. The present finding that reference strains of *Treponema pallidum* and *Borrelia burgdorferi* as well as the spirochetes found in the AD cases showed positive immunoreaction with a monoclonal antibody against APP, suggests that the APP may be an integral part of the infectious agent and thus may be the source of the excess of β A4 deposited in the AD brain. The fact that spirochetes of the *Borrelia burgdorferi* reference strain, cultured in a synthetic medium also exhibit a positive immunoreaction with the APP antibody may suggest that spirochetes synthesize their own APP.

In AD, β A4 is often deposited in the leptomeningeal and cortical vessel walls. In addition β A4 deposits were detected in tissues other than brain¹⁹ suggesting that AD is a systemic disorder. This would be in agreement with spirochetal invasion of the brain via the vascular system. Probably, as in syphilis, spirochetes may invade the CNS during the early stage of the disease. In a small proportion of patients infection takes place but may remain latent for periods of varying length. In general paresis, dementia may appear as long as 43 years after the primary infection.²⁰ Subsequently, the accumulation of the microorganisms in small clusters in the cortex may cause the formation of 'argyrophilic', 'senile' or 'neuritic' plaques while damage to the neurones may lead to neurofibrillary changes.

The clinical reports made no mention of signs or symptoms of meningitis, encephalitis or polyradiculoneuritis, nor were histological signs of meningitis or encephalitis in the 14 AD cases investigated here. Spirochetes may invade the parenchyma of several organs including the brain without the challenge of an inflammatory reaction.¹⁴ However, we cannot exclude the possibility that the amyloid-bearing plaques may be the sites of a chronic inflammatory process.²¹

There were no Alzheimer-like changes in the 13 age matched control cases investigated in this study. It is well known that in the older population there are cases demonstrating a small number of plaques and neurofibrillary tangles. These cases where the number of plaques and tangles with respect to the age of the patients²² are not sufficient for the neuropathological diagnosis of AD, are said to reflect 'normal ageing'. In the cortex of these cases one may expect to find spirochetes in a small or moderate number. This was not seen in any of the 13 control cases (with use of the silver

techniques for AD, or with the immunohistochemical technique for the demonstration of β A4), probably because of the restricted number of cases investigated. When a larger, particularly an aged, population without AD is tested, one may well find spirochetes in the blood in a number of cases but not in the CSF and in cerebral cortex. In a number of AD cases spirochetes may well be present in the CSF and in the cerebral cortex but not in the blood.

Recently the microorganism, Actinomyces, has been reported to be present in the brain of patients with an incidence that was four times higher than in other pathological or normal conditions.²³ Gram staining or ultrastructural analysis did not reveal the presence of Actinomyces in the AD cases presented here.

It was demonstrated that the messenger RNAs encoding the three major APPs, are present in many tissues besides brain in both control and AD cases, suggesting that APP is a natural constituent of a variety of cells.^{24,25} The finding that the APP gene resides also on chromosome 21,²⁶ suggested the possibility that the APP gene, as the site of mutations, may cause AD. This hypothesis was subsequently challenged, when further studies revealed that the AD locus and the APP gene are not linked and not coinherited in familial cases of AD.^{27,28}

Few familial AD cases, representing only a very small proportion of all AD cases (familial and sporadic cases together) are caused by a genetic defect.²⁹⁻³¹ The similar localization and distribution of the β A4 deposited in the brain in both forms—familial and sporadic—are difficult to explain. An alternative reconciliation of the genetic and infectious aetiology of AD lies in the supposition that the genetic defects associated with AD may lead to a predisposition for spirochetal infection, or may favour its progress.

Conclusion

In conclusion, the isolation of spirochetes from all the 14 AD cases investigated suggests that AD may correspond to a tertiary stage of neurospirochetosis. The fact that spirochetes isolated from AD brains, as well as reference strains of *Treponema pallidum* and *Borrelia burgdorferi*, express positive immunoreactivity with a specific antibody against APP would seem to indicate that several types of spirochetes may contribute to the aetiology of AD. An immunohistochemical and ultrastructural analysis that demonstrated a similar localization of APP, of β A4 and of spirochetes in the brain of a patient with concurrent AD and Lyme disease, would appear to support this hypothesis.

The characterization of the spirochetes found in AD is now needed. Knowing the genus and species of these spirochetes would indicate the source, the mode of

transmission, and the site of the primary infection. It would enable one to develop serological tests for early detection of the infection. The pathological process is thought to begin long before the diagnosis of 'dementia' is made, and thus, appropriate antibiotic treatment should start early in order to prevent the development of dementia.

The presence of histological signs of AD in the brain, even in small numbers, would signify that the illness is in progress. At all times the late latent stage may turn into tertiary clinical manifestations.²⁰ In this light, the use of quantitative criteria for the neuropathological diagnosis of AD is unjustified.

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